National Aeronautics and Space Administration

Engineering of Methane Metabolism in *Pichia pastoris* through Methane Monooxygenase Expression

Samantha T. Fleury,[1,2] Lily S. Neff,[3,4] Jonathan M. Galazka[5]

[1]Universities Space Research Association, NASA Ames Research Center, Moffett Field, CA; [2]Department of Biology, University of Virginia, Charlottesville, VA; [3]Space Life Sciences Training Program (SLSTP), NASA Ames Research Center; [4]Department of Biological Chemistry, Wesley College, Dover, DE; [5]Space Biosciences Division, NASA Ames Research Center.

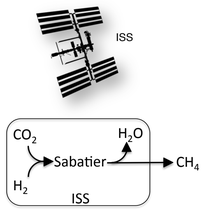
Background

Utilization of available resources is important to minimize the need for costly resupply from Earth. Currently the oxygen reclaiming Sabatier system on the ISS reacts CO_2 and H_2 to form H_2O and CH_4. The water is recycled back into the ISS system, but the methane is vented into space as waste. One potential use for this methane is as a carbon substrate for a biological production platform such as the methylotrophic yeast, *Pichia pastoris*. *P. pastoris* is a well-established synthetic biology platform and its native methanol metabolism is one enzymatic step away from metabolizing methane. In methanotrophic bacteria that step is carried out by methane monooxygenases (MMOs), which oxidize methane to methanol. In this project, we have attempted to engineer methane metabolism into *P. pastoris* by expressing a bacterial MMO system.

Sabatier produces methane

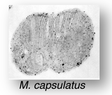
- The ISS Sabatier system produces methane and water from CO_2 and H_2.
- Water is recycled but CH_4 is vented to space.
- CH_4 could feed heterotrophic microbes for in-space bio-manufacturing.

Astronaut installing Sabatier system

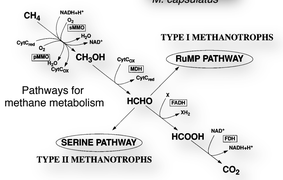


Natural methanotrophs have limitations

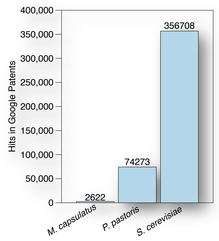
- Natural methanotrophic (consume methane) bacteria exist and are being developed as microbial factories.
- They utilize Methane Monooxygenases to hydroxylate methane to methanol.
- These microbes have limited engineering tools available and innovation is relatively slow.



M. capsulatus

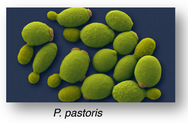


Pathways for methane metabolism

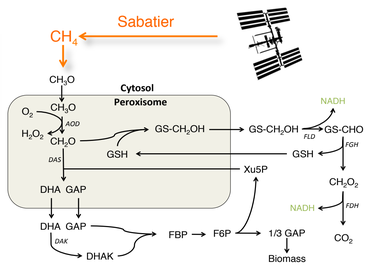


Pichia pastoris as a synthetic methanotrophic yeast

- *P. pastoris* is well-established methylotrophic (consumes methanol) yeast
- Used to produce Trypsin, murine TNFα, and FDA approved drugs Kalbitor and Jetrea.
- Addition of Methane Monooxygenase should allow growth on methane.



P. pastoris



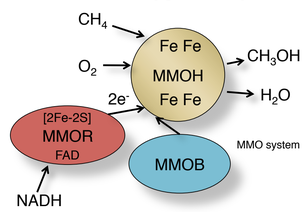
Methanol metabolism by *P. pastoris* and addition of methane metabolism

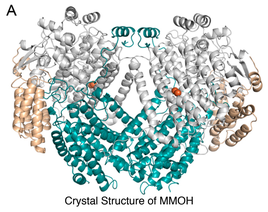
CO_2 → Sabatier → CH_4 → MMO → Pichia → Product

www.nasa.gov

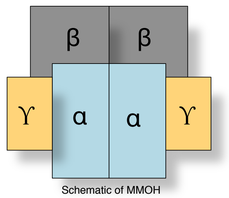
Results

Methane monooxygenase system





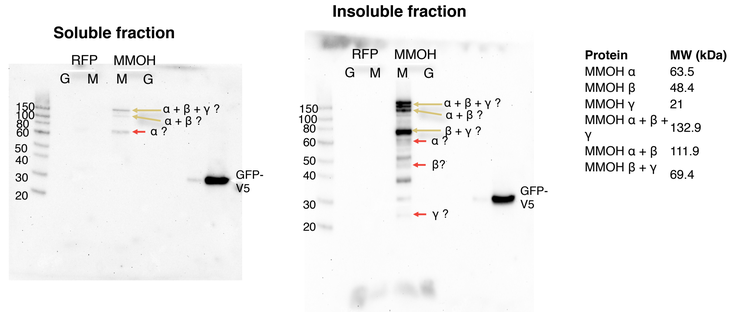
Crystal Structure of MMOH



Schematic of MMOH

- Methane hydroxylation in bacterial methanotrophs is carried out by MMOH, a hexamer of 3 polypeptides (α, β, ϒ).
- MMOR, a reductase is, transfers electrons to MMOH and is also required.
- MMOB may be important to speed the catalytic cycle.
- Assembly of MMOH into the correct 3D structure is required and may not readily occur outside of native host.

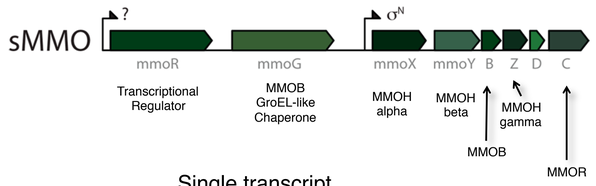
Methane monooxygenase expression

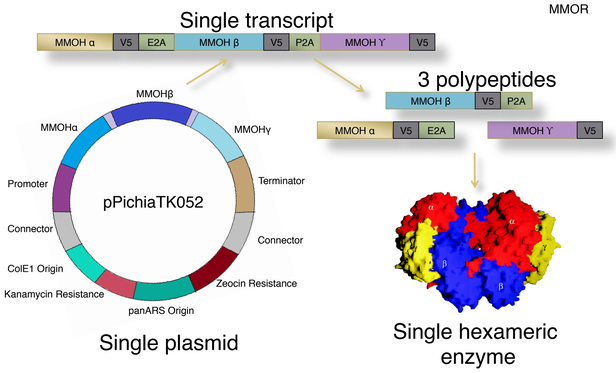


Protein	MW (kDa)
MMOH α	63.5
MMOH β	48.4
MMOH ϒ	21
MMOH α + β + ϒ	132.9
MMOH α + β	111.9
MMOH β + ϒ	69.4

- Bands corresponding to MMOH subunits were found only in the lane from *P. pastoris* expressing the MMOH plasmid grown on methanol as expected for a gene expressed under a methanol induced promoter.
- Unexpected bands suggest incomplete skipping by type 2A peptides

Balancing MMOH subunit concentration

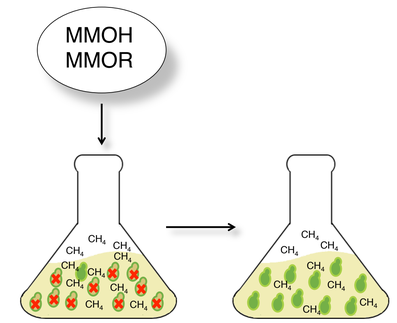




- MMOH subunits are expressed from a single operon in methanotrophic bacteria suggesting that balanced subunit concentration is important
- To mimic this in *P. pastoris* we built plasmid where MMOH subunits are expressed on a single transcript that includes type 2A "skipping sequences".
- During translation of this transcript the ribosome should skip a peptide bond after the 2A sequences resulting in 3 polypeptides at equal concentration.

Testing minimal system

- Expression and assembly of MMOH is not optimal.
- Regardless, we have created a strain containing MMOH and MMOR, which contains all components necessary for *P. pastoris* growth on methane.
- We can now set up a powerful selection for a functional system by growing on strain in media containing methane as a sole carbon source.



Conclusions

Engineering *P. pastoris* to metabolize methane offers one way to utilize currently wasted methane. To engineer *P. pastoris* we have created new engineering tools including promoters to work in *P. pastoris* and shown that they are functional based on their ability to drive expression of RFP. Preliminary data suggests that *P. pastoris* is capable of expressing MMOH, but further testing needs to be done to confirm expression and functionality. While completing this testing we are also moving forward with engineering expression of other proteins in the MMO system, with the goal of ultimately growing engineered *P. pastoris* on a methane substrate for functional testing.

References

Lee, Michael E., DeLoache, William C, Cervantes, Bernardo, Dueber, John E. (2015) A Highly Characterized Yeast Toolkit for Modular, Multipart Assembly. *Acs Synth. Biol.* 4, 975-986.

Liang, Shuli et al. (2012) Comprehensive structural annotation of Pichia pastoris transcriptome and the response to various carbon sources using deep pair-end RNA sequencing. BMC Genomics 13,738.

Sirajuddin, Sarah, Rosenzweig, Amy C. (2015) Enzymatic Oxidation of Methane. Biochemistry 54, 2283.